Histone lysine methylation is a reversible process, dynamically regulated by both lysine methyltransferases and demethylases. In particular, histone H3 lysine 9 (H3K9) trimethyl (H3K9me3) is associated with active transcription, whereas methylation of H3K79, H3K27, or H3K4R20 is associated with gene silencing. EZH2 is a highly conserved histone methyltransferase that specifically targets H3K27 and functions as a transcriptional repressor (Nguyen et al., 2011). Tissue microarray analysis of breast cancers identified consistent overexpression of EZH2, which was strongly associated with tumor aggressiveness. The compound DZNep has been shown to inhibit EZH2, decrease methylation of H3K27, and selectively induce apoptosis of cancer cell lines, including MCF-7 (Tan et al., 2017).

Histone lysine demethylase 1 (SDLI1) is the first identified histone lysine demethylase capable of specifically demethylating monomethylated and dimethylated lysine 4 of histone H4 (H4K3me1 and H4K3me2) (Huang et al., 2011). DSLI1 is highly expressed in ER-negative breast tumors, and hence DSLI1 was selected to serve as a predictive marker for aggressive breast tumor biology and a novel attractive therapeutic target for treatment of ER-negative breast cancers. DSLI inhibitor has been shown to inhibit DSLI and provides a mechanism of demethylating of H4K3 and thus release gene silencing, as well as promoting toxicity of cancer cells (Konovolov et al., 2017).

Enhanced activity of histone-modifying enzymes such as LSD1 and EZH2 leads to epigenetic silencing of critical genes, such as tumor suppressor genes, that have been shown to play an important role in breast tumor tumorigenesis. A series of novel compounds which function as powerful inhibitors of histone methylation or demethylation are capable of inducing re-expression of aberrantly silenced genes important in breast tumorigenesis.

Here we demonstrate the ability to monitor the effect of histone demethylase and demethylase inhibitors that selectively induce apoptosis in cancer cell lines. MCF-7 breast cancer cells stably expressing EZH2 were cultured in medium with or without selective concentrations of DZNep and DSLI inhibitor, and the presence of calcium. In apoptosis, phosphatidylserine (PS) is translocated to the outer leaflet of the plasma membrane. The appearance of PS on the cell surface is a universal indicator of the initial/intermediate stages of apoptosis and can be detected before morphological changes can be observed. PS is also a valuable probe to detect apoptotic cells which have expressed PS on the cell surface. The 347 nm and 443 nm excitation and emission maxima of the 350 Conjugate (AAT Bioquest Catalog No. 20070) are compatible with the Cell Imaging Multi-Mode Reader. Cytation 5 includes filter- and fluorescent excitation and emission maxima of the 350 Conjugate (AAT Bioquest Catalog No. 20070) are compatible with the Texas Red imaging filter cube of the Cytation 5.

Phenotypic Cytotoxicity Assessment

The ability to induce an eventual toxic effect in the target cell type by potential new anti-cancer therapies is critical. However, it is also important to incorporate an appropriate cell model. Tumors are made of multiple cell types, including cancer and stromal cells. Therefore the inclusion of human dermal fibroblasts can create a more appropriate microenvironment for testing. The use of cells expressing fluorescent proteins has become increasingly popular for the identification of the effect that a molecule has on each specific cell type. DZNep, Chaetocin, and LSD Inh. 1, all known to modify the state of histone lysine methylation, were tested to ascertain whether cytotoxicity was induced in co-cultured cell types over the total dosing period.

Cytological Cytotoxicity Assessment

Cytation 5 is a modular multi-mode microplate reader that combines automated digital microscopy and microplate detection. Cytation 5 includes filter- and monochromator-based microplate reading, the microscopy module provides up to 64 magnification in fluorescence, brightfield, phase, and time-lapse microscopy. Cytation 5 cell culture temperature control to 65 °C, 75 °C, or 60 °C, CO2, gas control and dual injectors for kinetic assays. Shaking and DMSO or DMSO-free media are also standard. The instrument was used to image cells expressing fluorescent proteins, in addition to fluorescent probes and labeled 2′ Ab. Following compound incubation periods.

Epigenetic Mechanism of Action Determination

The combination of phenotypic analysis with mechanism of action determinations has seen increasing adaptation within the drug discovery and preclinical development pipeline of the effects of test molecules, including epigenetic inhibitors. The incorporation of primary antibodies specific for methylation states of particular histone lysine residues, coupled with fluorophore labeled secondary antibodies, present a sensitive and specific method to detect potential epigenetic modifications. The fact that these modifications take place within the nucleus of the target cell also allows the incorporation of a nuclear dye, and sub-population analyses performed by Gen5 Data Analysis Software.

4. Epigenetic Mechanism of Action Determination

The combinatorial analysis of cell lines expressing fluorescent proteins (FP) allows for simplified, rapid detection of the cytostatic effect of co-cultured cell models via the wide-field microscopy capability of the Cytation 5. Alternatively, fluorescence microscopy can be used to determine the cytostatic effect of multiple test compounds, such as the combinatorial compound mixture (Figure 2C and D). Inhibition of co-cultures was determined using fluorescent probes, while the photoproteins allowed differentiation of the final cytotoxic effect on the two cell types in the co-culture. Mechanism of action studies of the inhibitors were then performed using antibodies to the specific histone H3 lysine residues and their various states.

Cell Plating

Inhibitor Cytotoxicity Assessment: For cytotoxicity assays number measurements, MCF-7 breast cancer cells expressing GFP and human dermal fibroblasts expressing RFP were added in a volume of 100 µl of eight columns of multiple well-96 black, clear bottom-coated plates (Corning Life Sciences Catalog No. 356649) to create cell densities of 10,000 cells/well for each cell type. The procedures were also performed with apoptosis/necrosis assessments, with the exception that MCF-7 GFP cells were only added to the plate.

Epigenetic Mechanism of Action Determination: The same procedures as previously described was performed, with the exception of non-FP expressing MCF-7 cells alone being used in the experiment.

Compound Dosing

Following an overnight incubation at 37 °C/5% CO2, medium was removed and replaced with either medium containing Hoechst 33342 for cytotoxicity/cell number measurements, or Annexin V-iFluor™ 350 Conjugate and 7-AAD fluorescent probes for apoptosis/necrosis assessments. Imaging was then performed with the Cytation 5 using 4x or 20x objectives, followed by cellular analysis using Gen5™ Data Analysis Software.

Epigenetic Mechanism of Action Determination: Upon completion of the incubation period, compound containing medium was removed and replaced with either medium containing Hoechst 33342 for cytotoxicity/cell number measurements, or Annexin V-iFluor™ 350 Conjugate and 7-AAD fluorescent probes for apoptosis/necrosis assessments. Imaging was then performed with the Cytation 5 using 4x or 20x objectives, followed by cellular analysis using Gen5™ Data Analysis Software.

Determination of apoptotic and necrotic activity leading to cytotoxicity can also be performed with the addition of fluorescent probes (Figure 2). Chaetocin induces both apoptosis and necrosis within MCF-7 cells in a time and dose dependent manner (Figure 2A and B). DZNep exerts a more controlled effect on the cells, activating apoptotic pathways within cells (Figure 2C). Chaetocin was added to the Cytation 5 in a dose and concentration dependent manner (Figure 2), while DZNep was added to the Cytation 5 in a dose and concentration dependent manner (Figure 2A). Inhibition of cell viability was determined using fluorescent probes for apoptosis (Figure 2 and 3).

The results shown above validate the mechanism of action exhibited by the test compound, and also confirm the ability of the Cytation 5 to detect modifications in the epigenetic state of treated and untreated cancer cell models.

Conclusions

1. The combination of cell lines expressing fluorescent proteins (FP) allows for simplified, rapid detection of the cytostatic effect of co-cultured cell models via the wide-field microscopy capability of the Cytation 5 and individual imaging channels.

2. Live cell assays can also be multiplexed with FP expressing cells to further understand the phenotypic effects of test molecules, using Cytation 5 and individual imaging channels.

3. Cellular analysis to determine live, apoptotic, and necrotic cell numbers following prescribed incubation periods is carried out using Gen5 Data Analysis Software.

4. Assessment of modifications to the methylation state of target histone lysine residues are also possible using antibody-based detection, and the sub-population capabilities of Gen5.